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Alternative Lactose Catabolic Pathway in *Lactococcus lactis* IL1403

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In this study, we present a glimpse of the diversity of *Lactococcus lactis* subsp. *lactis* IL1403 β-galactosidase phenotype-negative mutants isolated by negative selection on solid media containing cellobiose or lactose and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and we identify several genes essential for lactose assimilation. Among these are *ccpA* (encoding catabolite control protein A), *bglS* (encoding phospho-β-glucosidase), and several genes from the Leloir pathway gene cluster encoding proteins presumably essential for lactose metabolism. The functions of these genes were demonstrated by their disruption and testing of the growth of resultant mutants in lactose-containing media. By examining the *ccpA* and *bglS* mutants for phospho-β-galactosidase activity, we showed that expression of *bglS* is not under strong control of CcpA. Moreover, this analysis revealed that although BglS is homologous to a putative phospho-β-glucosidase, it also exhibits phospho-β-galactosidase activity and is the major enzyme in *L. lactis* IL1403 involved in lactose hydrolysis.

Bacteria have evolved three different systems for the assimilation of the main milk sugar, lactose, which differ in their phosphorylation states, intermediate metabolites, and bioenergetics. They include the group translocation systems (13, 39) and the primary (15) and secondary (27, 37) transport systems.

During transport by the bioenergetically most efficient group translocation system, the lactose-specific phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) (*lac*-PTS), lactose is phosphorylated at the C-6 carbon, and the internalized lactose 6-phosphate is degraded into galactose 6-phosphate and glucose by phospho-β-galactosidase. It has been suggested that in some cases lactose 6-phosphate can be hydrolyzed by β-glycosidases specific for β-glucoside sugars, that is, by P-β-glucosidases (46). This seems to be supported by sequence similarities between P-β-galactosidase and P-β-glucosidase enzymes, both of which, according to the nomenclature of Henrissat (24), belong to family I of glycohydrolases.

In primary and secondary transport systems lactose is not phosphorylated, and after internalization, it is hydrolyzed by β-galactosidase, yielding glucose and galactose. Galactose is subsequently metabolized through the Leloir pathway. Uptake of lactose via primary transport systems depends on hydrolysis of ATP, which provides energy for translocation of the substrate by an ATPase. Secondary transport systems use the energy from solute gradients, and in sugar translocation different types of mechanisms are involved, such as symport, antiport, and uniport.

Lactococcus lactis is a lactic acid bacterium (LAB) used in the dairy industry as a starter culture. Some strains of this species are able to ferment lactose present in milk very rapidly. In these strains lactose is transported by the *lac*-PTS and hydrolyzed by P-β-galactosidase. The galactose 6-phosphate formed is further metabolized via the tagatose 6-phosphate pathway. In lactococci, operons encoding proteins involved in efficient lactose transport and metabolism are located on plasmids. Because of this and wide biotechnological applications of lactococci, lactose-positive strains have been extensively studied over the past decades (14, 51). *lac*-PTS genes can also be located on chromosomes, as has been described for *Streptococcus mutans* (41) and the non-LAB *Staphylococcus aureus* (6, 7, 42). Moreover, there is some indirect evidence that additional *lac*-PTS genes can also be located in the genomes of several *L. lactis* strains (3, 9, 10, 12, 46).

Besides the *lac*-PTS, there is only one other type of lactose transport system that has been described for *L. lactis*, namely, the lactose-H^+ symport permease (26). Two other known types of lactose transport systems, the lactose-galactose antiporter and ABC protein-dependent lactose transporter, have been found in *Streptococcus thermophilus* (36, 37, 38) and in the non-LAB, gram-negative *Agrobacterium radiobacter* (57), respectively.

L. lactis IL1403, a plasmid-cured derivative of the IL594 strain (8), is essentially lactose negative and does not contain in its chromosome genes encoding proteins homologous either to PTS lactose permease or to P-β-galactosidase (4). Despite this, *L. lactis* IL1403 possesses another system that has been shown in several other bacteria to be involved in lactose assimilation, a lactose permease–β-galactosidase system. In IL1403 this system depends on proteins encoded by genes of the Leloir pathway operon: *lacS*, specifying a putative H^+-lactose symporter or galactose-lactose antiporter; *lacA*, coding for thiogalactoside acetyltransferase; and *lacZ*, encoding β-galactosidase. The other genes of the Leloir pathway cluster encode proteins involved in galactose catabolism, such as GalM, GalK, GalT, and GalE (4).

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Recently, it has been observed that in the presence of cellobiose *L. lactis* IL1403 shows a lactose-positive phenotype (1). The catabolite control protein A (CcpA), a member of the LacI-GalR family of bacterial repressors (55), has been shown to play a role in this phenomenon (1). CcpA acts as a global regulator of carbon catabolite repression (CCR) in low-GC gram-positive bacteria, and its regulatory functions have been characterized in detail in *Bacillus subtilis* (16, 23, 32, 33, 34). To carry out its role, CcpA interacts with the corepressor, a serine-phosphorylated phosphocarrier protein (P-Ser_{46}-HPr) (16). HPr is one of the proteins constituting the PEP-PTS, and after phosphorylation at its His-15 position by enzyme I (P-His_{15}-HPr), it is involved in the transfer of the phosphoryl group to the sugar-specific enzyme II (39). CcpA in complex with P-Ser_{46}-HPr has been shown to exert its function by binding to a *cis*-acting DNA site called the catabolite-responsive element (*cre*) (54), located in front of or within the CcpA-regulated genes and operons. CcpA alone also interacts with DNA, but this binding is nonspecific and very weak (32). In most cases CcpA acts as a repressor (22, 34), but some examples showed it also to mediate catabolite activation (19, 50).

In this paper we describe the identification of several mutants of *L. lactis* IL1403 that exhibit a β-galactosidase-negative phenotype in the presence of cellobiose or lactose and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We show that non-PTS-specific BglS and LacS proteins are involved in lactose and X-Gal utilization, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *L. lactis* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *E. coli* was cultivated at 37°C in Luria-Bertani (LB) medium to which 1.5% agar (Merck) or erythromycin (Em) or ampicillin (Amp) (100 μg/ml) was added when necessary. The *L. lactis* strains were grown at 30°C in M17 broth (Difco Laboratories, Detroit, MI) or in a synthetic chemically defined medium (CDM) (35, 40) containing 1% of glucose (G-M17 or G-CDM), lactose (L-M17 or L-CDM), cellobiose (C-M17 or C-CDM), or galactose (Gal-CDM), as required. The use of CDM instead of the rich M17 eliminates the "M17 effect," that is, residual *L. lactis* growth even in the absence of any added sugar.

Determination of β-galactosidase-positive phenotype. For the induction of the β-galactosidase-positive phenotype, *L. lactis* strains were cultivated in L-M17 or L-CDM broth with cellobiose added at the inducible concentration of 0.01% (CL-M17 or CL-CDM). β-Galactosidase activity in *L. lactis* was detected by adding 80 μg/ml of X-Gal to C-M17, L-M17, C-CDM, or L-CDM agar plates. β-Galactosidase activity of *E. coli* colonies was detected on LB agar plates containing 50 μg/ml of X-Gal and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Colonies of cells with a β-galactosidase-positive phenotype developed a blue color.

DNA manipulation and transformation. Molecular cloning, restriction enzyme analysis, and transformation of *E. coli* were performed according to general procedures (43). *L. lactis* cells were made competent and used for electroporation with pGhost9::IS*S1* as described by Holo and Nes (25) and Wells et al. (56). Transformants were selected on G-M17 agar plates containing 5 μg/ml Em, at 30°C, after 2 h of regeneration in G-M17-saccharose (0.5 M) medium. Plasmid DNAs from *E. coli* and *L. lactis* strains were isolated using QIAGEN columns and protocol with the modification for *L. lactis* that 5 mg/ml lysozyme was added to P1 buffer. Restriction and modifying enzymes were purchased from Fermentas (Lithuania) and used according to the recommendations of the manufacturer.

Plasmid integration mutagenesis, Southern hybridization, and DNA rescue-cloning. Chromosomal DNA of *L. lactis* IL1403 was randomly mutagenized by integration of the pGhost9::IS*S1* plasmid as described previously (30). To validate this method, three independent mutagenesis procedures were performed. Mutagenized cells were plated on M17 agar plates containing X-Gal, Em, and 1% of cellobiose or 1% of lactose and grown for 3 days at 37.5°C. Mutants forming white colonies, indicating their β-galactosidase-negative phenotype, were then selected.

To identify genes surrounding the pGhost9::IS*S1* integration site, total DNA was isolated from *L. lactis* IL1403 cells growing exponentially in G-M17 with Em as described previously (21). EcoRI- or HindIII-generated DNA fragments (2 μg) were separated on 0.8% agarose gels and transferred onto a nylon Hybond-N+ membrane (Amersham International, United Kingdom) (43). Fluorescent probe labeling, hybridization, and detection were done with the ECL labeling and detection procedures (Amersham). The DNA probe containing the IS*S1* sequence comprised a 1,037-bp fragment amplified from pGhost9::IS*S1* by using the uni and pGh9 primers (Table 1).

The DNA rescue-cloning procedure applied was as described previously (1, 30). Briefly, total DNA isolated from mutants was cut with EcoRI or HindIII, self-ligated, and cloned in *E. coli* EC1000 (28). Clones containing pGhost9::IS*S1* linked to its flanking chromosomal DNA fragments (EcoRI [left] or HindIII [right]) were selected on LB plates containing Em. Rescued fragments were sequenced directly from pGhost9::IS*S1* by using the dideoxynucleotide chain termination method (42, 44) with primers pIS*S1*H and pIS*S1*E (Table 1).

The sequences obtained were analyzed and compared to the *L. lactis* IL1403 genome sequence database (4) by using the National Center for Biotechnology Information BLAST network service and standard parameters (2).

Stabilization of the *L. lactis* IL1403 *ccpA* mutant. In our previous study (1), the *ccpA* mutant (IBB550pGh) and the nucleotide sequence of the *ccpA* gene (GenBank accession number AF106673) were obtained by applying pGhost9::IS*S1* mutagenesis (30). Since pGhost9::IS*S1* possesses a thermosensitive replicon, this mutation could be stably maintained only at the nonpermissive temperature of 37°C. As this temperature is not physiological for lactococci, the pGhost9::IS*S1*-generated mutation was stabilized by excision of the integrative plasmid, leaving a single copy of the IS*S1* in the plasmid insertion site (30). Southern hybridization and DNA sequencing were used to verify the correct chromosomal structure of the stable *ccpA* mutant, named IBB550.

Enzyme assays. *L. lactis* cells were grown overnight at 30°C in CDM containing the appropriate sugar. A sample (20 ml) of culture was collected by centrifugation and resuspended in 1 ml Z buffer (31). Crude extracts were obtained by vortexing three times for 1 min each at high speed (Mini Bead Beater MBB-8) with glass beads (106-μm diameter; Sigma) at 1-min intervals, during which the cells were kept on ice. Cellular debris and glass beads were removed by centrifugation for 10 min at 8,000 rpm. The activities of β-glycosidases in cell extracts were determined by using the chromogenic substrates *p*-nitrophenyl β-D-glucopyranoside, *o*-nitrophenyl β-D-glucopyranoside, *o*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-gentiobioside, and *o*-nitrophenyl β-D-cellobioside. P-β-galactosidase activity was assayed with *o*-nitrophenyl β-D-galactopyranoside-6-phosphate at 30°C, as described by Miller (31). All substrates were obtained from Sigma. As a control for enzyme specificity, *p*-nitrophenyl α-D-galactopyranoside-6-phosphate and *p*-nitrophenyl α-D-glucopyranoside-6-phosphate (kind gifts from J. Thompson) were used. Protein concentrations were determined using the Bio-Rad protein assay (5).

Sugar utilization. Growth tests were performed in CDM supplemented with various sugars, using a Microbiology Reader Analyser (Bioscreen C; Labsystems, Finland). Changes in absorbance of the culture at 600 nm were monitored during growth at 25-min intervals. Additionally, sugar fermentation patterns were determined using the API 50CH test as specified by the manufacturer (API-BioMerieux, Marcy l'Etolie, France) after 3, 6, and 48 h of incubation.

Construction of *ccpA*, *bglS*, *galK*, *galT*, *galE*, and *lacZ* mutants through disruption with pJIM2374. Mutants were created by single crossover between pJIM2374 harboring the respective internal DNA fragment of the *ccpA*, *bglS*, *galK*, *galT*, *galE*, or *lacZ* gene and the chromosomal copy of each of these genes. The internal fragments were amplified using the appropriate "for" and "rev" primer pairs (Table 1). The PCR-generated DNA fragments were then ligated with the pGEM-T vector and transferred into *E. coli* TG1. The resultant plasmids were isolated from Amp^r cells that were unable to hydrolyze X-Gal. The orientation of inserts in pGEM-T was checked with the primer 1224 and the appropriate rev primer. Next, isolated plasmid DNA was digested with SalI and SacI, followed by ligation in pJIM2374 digested with the same restriction enzymes and transformation of *E. coli* TG1. The resulting hybrid plasmids comprising pGEM-T, pJIM2374, and internal fragments of the appropriate genes were isolated. The pGEM-T was removed by excision with NcoI, and the rest was self-ligated and transformed into an *E. coli* RepA strain, because pJIM2374 lacked replication functions. Subsequently, the plasmids were isolated from the resultant Em^r transformants and transferred into the *L. lactis* wild-type strain. Homologous recombination was enforced by 10^{-5} dilution in fresh G-M17–Em medium of the overnight culture of the lactococcal strain harboring pJIM2374 with the internal fragment of the appropriate gene. Diluted cultures were incubated for 3 h in G-M17 at 37°C. Integrants containing pJIM2374 in the appropriate gene in the chromosome were selected at 37°C on G-M17 agar plates

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer pair	Relevant properties	Source and/or reference[a]
Strains		
L. lactis		
IL1403	Lac$^-$, plasmid-free wild type, host strain	INRA (8)
IBB550pGh	Lac$^+$, CcpA$^-$, Em^r (pGhost9::IS*S1* integrant), plasmid-free, IL1403 derivative	IBB (1)
IBB550	Lac$^+$, CcpA$^-$, Em^s, plasmid-free, IL1403 derivative	This study
ccpA mutant	IL1403 derivative, Em^r, P*ccpA*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *ccpA* gene interrupted by pJIM2374 integration	This study
bglS mutant	IL1403 derivative, Em^r, P*bglS*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *bglS* gene interrupted by pJIM2374 integration	This study
galK mutant	IL1403 derivative, Em^r, P*galK*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *galK* gene interrupted by pJIM2374 integration	This study
galT mutant	IL1403 derivative, Em^r, P*galT*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *galT* gene interrupted by pJIM2374 integration	This study
galE mutant	IL1403 derivative, Em^r, P*galE*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *galE* gene interrupted by pJIM2374 integration	This study
lacZ mutant	IL1403 derivative, Em^r, P*lacZ*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *lacZ* gene interrupted by pJIM2374 integration	This study
bglS ccpA mutant	IBB550 derivative, Em^r, P*bglS*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *bglS* gene interrupted by pJIM2374 integration	This study
galK ccpA mutant	IBB550 derivative, Em^r, P*galK*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *galK* gene interrupted by pJIM2374 integration	This study
galT ccpA mutant	IBB550 derivative, Em^r, P*galT*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *galT* gene interrupted by pJIM2374 integration	This study
galE ccpA mutant	IBB550 derivative, Em^r, P*galE*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *galE* gene interrupted by pJIM2374 integration	This study
lacZ ccpA mutant	IBB550 derivative, Em^r, P*lacZ*::*luxAB* chromosomal transcriptional fusion with concomitant inactivation of the *lacZ* gene interrupted by pJIM2374 integration	This study
E. coli		
EC1000	Km^r, RepA$^+$ MC1000, carrying a single copy of the pWV01 *repA* gene in the *glgB* gene	RuG (28)
TG1	*supE* Δ*thi*(*lac-proAB*) *hsdD5* (F′$^+$ *traD36 proAB lacI*^{q}Z*ΔM15*)	17
Plasmids		
pGEM-T	Amp^r, M13*ori*, linear T-overhang vector	Promega
pGhost9::IS*S1*	Em^r, *repA*(Ts), IS*S1*	INRA (30)
pJIM2374	Em^r, integrative vector carrying the *luxAB* genes	INRA (11)
Primer pairs		
*ccpA*for/*ccpA*rev	CGCGCCAGAAGGTCTTAGAA/GACGCATTGCTACTGCTCC	L0143
*pepQ*for/*trxB*rev	CAGTATAGCTGAAGGAACAGTAAG/CCAACAGGACCTGCTCCAAC	L96847/L00196
*bglS*for/*bglS*rev	GGATGTCGCTGTTGATC/CTCAAGCCTTACGGGTC	L179659
*galK*for/*galK*rev	GGAACAACAGGACTAGCTCGTC/GCAACAAGAGCGATTGCACAAC	L0028
*galT*for/*galT*rev	GAGCCACTTGATGAGCTC/CGAATTCGAGCAATCGG	L0027
*galE*for/*galE*rev	GGTGGAGCAGGATACGTG/CATCTGGGTCTCCTGCAC	L0024
*lacZ*for/*lacZ*rev	GGTTGCCAATGCATACACC/CGGAGTCATTCCATGCG	L0025
uni/pGh9	GTAAAACGACGGCCAGT/GACAGCTTCCAAGGAGC	
pIS*S1*H/pIS*S1*E	AGCTTAAGAACAAGAAGG/CTAAAATAGACTTATCAG	
1224	CGCCAGGGTTTTCCCAGTCACGAC	

[a] INRA, Institut National de la Recherche Agronomique (Jouy-en-Josas, France); IBB, Institute of Biochemistry and Biophysics (Warsaw, Poland); RuG-MG, Department of Molecular Genetics, University of Groningen, (Groningen, The Netherlands).

containing Em. The physiological effect of gene disruption in the resultant mutants was examined.

Replacement of the *ccpA* mutated gene with its wild-type copy. In order to complement the *ccpA* mutation of strain IBB550, the *ccpA* gene with its putative promoter region was amplified using *pepQ*for and *trxB*rev primers (Table 1), cloned into pGEM-T, and transferred into *E. coli* TG1. The resultant plasmid DNA was isolated, digested with SalI, ligated to pJIM2374 digested with the same restriction enzyme, and transferred into *L. lactis* IBB550.

RESULTS

Cellobiose induces a β-galactosidase-positive phenotype in the lactose-negative *L. lactis* strain IL1403. Our previous results have shown that *L. lactis* IL1403 plated on M17 agar medium supplemented with cellobiose and X-Gal forms blue colonies (1). Here we tested whether various other sugars, including monosaccharides, disaccharides, β-glucosides, pentoses, and hexoses (ribose, galactose, glucose, fructose, mannose, mannitol, sorbitol, *N*-acetyloglucosamine, arbutin, esculin, salicin, maltose, lactose, saccharose, trehalose, rafinose, melobiose, xylose, arabinose, and glycerol) could induce the same phenotype. None of the 20 sugars tested allowed *L. lactis* IL1403 to develop blue colonies on X-Gal plates, suggesting that only cellobiose induces a metabolic pathway that confers on the cells of lactose-negative *L. lactis* IL1403 a β-galactosidase-positive phenotype. It is thus tempting to speculate that a cryptic lactose utilization system in *L. lactis* IL1403 could be under the control of cellobiose.

To test this assumption, *L. lactis* IL1403 was cultivated in CDM broth supplemented either with a low (0.01%) concen-

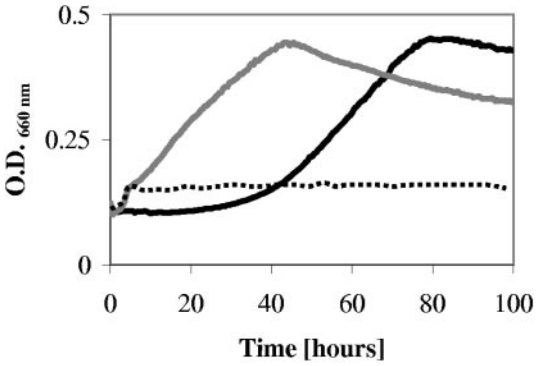

FIG. 1. Growth of *L. lactis* IL1403 in CDM supplemented with 1% lactose (black line), 0.01% cellobiose (dashed line), and 1% lactose with 0.01% cellobiose (gray line). $O.D._{660\ nm}$, optical density at 660 nm.

tration of cellobiose alone or with the same cellobiose concentration plus 1% lactose (Fig. 1). As a control, CDM supplemented only with 1% lactose was used. *L. lactis* could not grow in CDM with either 0.01% cellobiose or 1% lactose. However, after approximately 40 h of incubation the cells started to utilize lactose slowly, and they reached maximal absorbance after about 80 h. Addition to the L-CDM of a low concentration of cellobiose, which does not support growth of the strain, clearly led to induction of multiplication of *L. lactis* cells. *L. lactis* IL1403 formed white colonies on M17 agar plates containing glucose, cellobiose, and X-Gal, suggesting that catabolism of X-Gal undergoes catabolite repression. Altogether, these observations are indicative of cellobiose-inducible metabolic potential to assimilate lactose in the basically lactose-negative *L. lactis* strain IL1403.

Isolation of β-galactosidase-negative phenotype mutants of *L. lactis* IL1403 grown in C-M17. Previously, we have reported the isolation of *L. lactis* mutants that have lost the cellobiose-inducible β-galactosidase-positive phenotype (1). In that study, among about 2,000 pGhost9::IS*S1* integrants grown on C-M17 agar plates supplemented with X-Gal, 6 were found to be stably white. Here, using the same procedure, a second independent mutagenesis was performed, resulting in 31 white colonies on C-M17–X-Gal agar plates among 3,600 integrants.

To determine the number of different random transpositions of pGhost9::IS*S1* in the two mutagenesis procedures, total DNA was isolated from all 37 white integrants and analyzed by Southern hybridization using EcoRI and HindIII, enzymes that each cut once in the vector. In 5 out of 6 and in 29 out of 31 white integrants from each round of mutagenesis, different restriction patterns were found (data not shown). DNA regions flanking the plasmid integration site in the majority of these mutants were cloned and sequenced, and the sequences were compared to the *L. lactis* IL1403 genome database. Most insertions had occurred in different locations throughout the *L. lactis* chromosome (Table 2). Only *ccpA* (encoding the catabolite control protein A), and *yheB* (encoding a conserved hypothetical protein) were hit more than once (Table 2). Both in *ccpA* and in two of the four *yheB* mutants, independent pGhost9::IS*S1* insertions were found, which strongly indicates that the physiological changes in these mutants were, indeed, the result of inactivations of *ccpA* and *yheB*.

Derepression of sugar catabolism in the *ccpA* mutant. A stable *ccpA* mutant, *L. lactis* IBB550, was subjected to further studies. Utilization of sugars by strains IBB550 and IL1403 was investigated using the API 50CH test, under both aerobic and anaerobic conditions of growth. Among a set of 49 sugars, these strains were able to utilize to the same extent ribose, galactose, glucose, fructose, mannose, *N*-acetylglucosamine, cellobiose, maltose, and trehalose, although IBB550 began to assimilate arbutin, esculin, and salicin more rapidly than IL1403. Of the two strains, only IBB550 grew on lactose, gentiobiose, and amygdalin. The same differences in sugar utilization were observed under aerobic and anaerobic conditions of growth.

Since the medium utilized in the API 50CH test is rich and complex, we decided to also test the growth of IL1403 and IBB550 in synthetic CDM supplemented with glucose, cellobiose, lactose, and lactose with a 0.01% concentration of cellobiose (Fig. 2). Growth of strain IBB550 was 1.3-fold (0.49 ± 0.05/h versus 0.34 ± 0.03/h) and 1.25-fold (0.37 ± 0.05/h versus

TABLE 2. Proteins encoded in the *L. lactis* IL1403 genome matching the predicted products of DNA regions interrupted by pGhost9::IS*S1* integration in the β-galactosidase-negative mutants and their growth in L-CDM and CL-CDM media

Gene (hits)[a]	Function	Location on IL1403 chromosomal DNA	Growth on lactose[b]	
			With cellobiose[c]	Without cellobiose
ccpA (2)	Catabolite control protein A	1695930–1696928	++	++
yheB (4)	Conserved hypothetical protein	741571–742494	−−	−−
lacS (1)	Lactose permease		awt	−
ydgC (1)	Amino acid permease	361431–362810	awt	awt
ynhH (1)	Unknown protein	1378866–1379273	awt	awt
ydhB (1)	Hypothetical protein	371163–371975	awt	awt
ylfA (1)	Unknown protein	1150456–1151043	awt	awt
yrcA (1)	P-β-glucosidase	1721677–1723110	awt	awt
UP*fbp* (1)	Fructose-1,6-bisphosphatase	254043–255965	awt	awt
UP*topA* (1)	DNA topoisomerase 1	1254565–1256697	awt	awt
UP*ydhD* (1)	Hypothetical protein	373134–373424	awt	awt

[a] UP, integration of the plasmid occurred in the upstream noncoding region of the gene.
[b] awt, the mutant grows in the same manner as *L. lactis* IL1403 (its growth in CL-CDM was regarded as "+," and that in L-CDM was regarded as "+/−"); ++, very good growth; −, slight inhibition of growth; −−, lack of growth.
[c] Cellobiose was used at the inducing concentration of 0.01%.

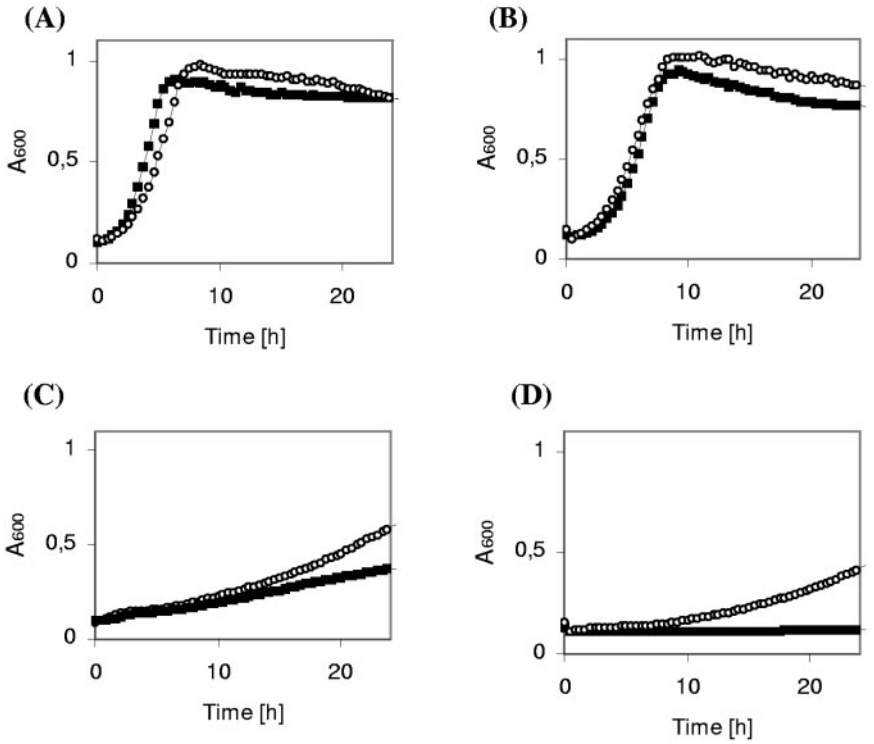

FIG. 2. Growth of IBB550 (*ccpA* mutant) (open circles) and *L. lactis* IL1403 (black squares) in CDM containing 1% glucose (A), 1% cellobiose (B), 1% lactose with 0.01% cellobiose (C), or 1% lactose (D).

0.29 ± 0.02/h) reduced on glucose (Fig. 2A) and cellobiose (Fig. 2B), respectively. In both strains cellobiose induced lactose catabolism, but the *ccpA*-deficient strain responded better, growing 1.5-fold faster (0.06 ± 0.002/h versus 0.04 ± 0.01/h) and showing a shorter lag phase than IL1403 (Fig. 2C). Unexpectedly, IBB550 was able to ferment lactose even in the absence of cellobiose (Fig. 2D), with a similar growth rate (0.06 ± 0.003/h).

The phenotype of IBB550 was also tested on lactose-containing M17 or CDM agar plates supplemented with X-Gal. Surprisingly, strain IBB550 was able to hydrolyze X-Gal in both media, resulting in blue colonies.

To test whether these phenotypic changes were indeed due to *ccpA* inactivation and not to an additional, unidentified mutation, the *ccpA* gene was disrupted by single-crossover recombination in the wild-type IL1403 strain. This mutant was found to exhibit the same properties as those described previously for IBB550 (data not shown). In addition, the *ccpA* mutation in IBB550 was complemented by the wild-type copy of *ccpA* cloned into pJIM2374. Strain IBB550(pJIM2374) regained the wild-type phenotype (data not shown).

P-β-galactosidase is elevated in *L. lactis* IBB550. The effect of the *ccpA* mutation on the enzymatic activities of β-galactosidase, P-β-galactosidase, β-glucosidase, P-α-galactosidase, and P-α-glucosidase was examined in *L. lactis* IL1403 and IBB550. P-β-galactosidase activity was detected in both strains. None of the other enzymes was detected in either of the strains grown in the presence of all the sugars tested.

The level of P-β-galactosidase activity in IL1403 was relatively low when it was grown in the presence of glucose (Table 3) and was 19-fold higher on cellobiose-lactose. P-β-galactosidase activity was only about threefold higher in IBB550 than in IL1403

TABLE 3. P-β-galactosidase activity in *L. lactis* IL1403 and its *ccpA* and *bglS* mutants grown in CDM supplemented with various sugars

Sugar[a]	Enzymatic activity (nmol/min/mg of protein)[b]			
	IL1403 (wild-type)	IBB550 (*ccpA*)	*bglS*	*bglS ccpA*
Glucose	10.1 ± 3.35	28.98 ± 1.14	3.5 ± 0.25	12 ± 1.02
Cellobiose-lactose	123.73 ± 15.69	180.02 ± 11.39	—	—
Lactose	—[c]	276 ± 26.7	—	—
Cellobiose	192.11 ± 19.16	243.64 ± 32.2	24.44 ± 3.14	26.32 ± 1.64
Galactose	19.95 ± 2.53	ND[d]	14.47 ± 0.98	ND
Galactose-cellobiose-lactose	51.06 ± 2.35	ND	10.05 ± 2.54	ND

[a] All sugars were used at the concentration of 1%; however, in media containing multiple sugars, the cellobiose concentration was 0.01%.
[b] Mean values and standard deviations from at least three independent experiments are given.
[c] —, unable to grow.
[d] ND, not determined.

TABLE 4. Multiple-hit DNA regions interrupted by pGhost9::IS*S1* integration in double β-galactosidase-negative phenotype mutants of the IBB550 (*ccpA*) strain

Region (hits)	Gene (hits)[a]	Function	Chromosomal localization of plasmid insertion	Growth on lactose[b]
I (8)	*galE* (1)	UDP-glucose 4-epimerase (EC 5.1.3.2)	2054147–2055127	−
	galT (1)	Galactose-1-phosphate uridylyltransferase (EC 2.7.7.10)	2058844–2060325	−
	galK (2)	Galactokinase (EC 2.7.1.6)	2060499–2061698	+/−
	galM (1)	Aldose 1-epimerase (EC 5.1.3.3)	2061725–2062744	+/−
	lacS (2)	Lactose permease		+/−
	yugA (1)	Transcription regulator	2064540–2065274	+/−
II (6)	*ydhB* (2)	Hypothetical protein	371163–371975	awt
	ydhC (4)	Hypothetical protein	371975–372952	awt
III (3)	*yfgQ* (3)	Hypothetical cation-transporting ATPase	568648–570975	awt
IV (3)	*yveH* (3)	Conserved hypothetical protein	2145997–2147083	+/−
V (3)	*pknB* (2)	Serine/threonine protein kinase	1955090–1956973	+/−
	pppL (1)	Serine/threonine protein phosphatase	1956973–1957638	+/−
VI (2)	*bglS* (2)	P-β-glucosidase A (EC 3.2.1.21)	179668–181104	−
VII (2)	UP*rheB* (1)	ATP-dependent RNA helicase	415804–417144	+/−
	UP*yebF* (1)	Hypothetical transcriptional regulator	417888–418634	−
VIII (2)	*yahA* (1)	Conserved hypothetical protein	72269–73081	+/−
	yahG (1)	ABC transporter ATP binding protein	73207–74844	+/−
IX (2)	*yhfB* (2)	Hypothetical protein	751095–753059	awt
X (2)	*yjiE* (2)	Conserved hypothetical protein	988807–989694	+/−
XI (2)	*yuaA* (1)	Hypothetical protein	2000601–2001545	ND[c]
	yuaD (1)	Conserved hypothetical protein	2004383–2005306	awt

[a] UP, integration of the plasmid occurred in the upstream noncoding region of the gene.
[b] Integrants were cultivated in L-CDM. awt, the mutant grows in the same manner as the parental, *ccpA*-deficient strain (its growth in L-CDM is regarded as "++"); +/−, impairment of growth; −, lack or severe inhibition of growth.
[c] ND, not determined.

grown in the presence of glucose and was six- to ninefold higher on cellobiose-lactose, lactose, or cellobiose.

Physiological characterization of the other *L. lactis* IL1403 β-galactosidase-negative phenotype mutants. To test whether the inability to hydrolyze X-Gal on C-M17 plates correlates with the expected inability to catalyze cellobiose-inducible fermentation of lactose, the 31 mutants obtained in the second round of pGhost9::IS*S1* mutagenesis were grown in CL-CDM. Unexpectedly, 27 of them were able to grow in CL-CDM in the same manner as the wild-type strain, whereas growth of the other four mutants was significantly impaired. These four mutants, which were unable to grow on lactose as the sole carbon source, were found to be inactivated in *yheB*. Another mutant, disrupted in *lacS*, retained full ability to grow in CL-CDM, but in comparison to IL1403 its capability of growth in L-CDM was reduced (Table 2).

Isolation of β-galactosidase-negative phenotype mutants in the *ccpA* background. Since the IBB550 strain exhibited an increased ability to hydrolyze lactose and loss of the cellobiose-inducible β-galactosidase phenotype (C-M17–X-Gal plate) while demonstrating a lactose-inducible β-galactosidase phenotype (L-M17–X-Gal plate), an attempt was made to elucidate which system is involved in this phenomenon. Therefore, IBB550 was randomly mutagenized using pGhost9::IS*S1*. Among the approximately 1,000 integrants grown on L-M17 plates supplemented with X-Gal, 100 were unable to hydrolyze X-Gal. Southern hybridization was used to show that 51 of these mutants had undergone a unique integration of pGhost9::IS*S1* (data not shown), and these clones were further analyzed by sequencing of insertion sequence extremities and by growth tests.

Eleven DNA regions in strain IBB550 were hit more frequently, strongly indicating their possible involvement in lactose and/or X-Gal metabolism (Table 4). In seven of these, IS*S1* had inserted into DNA regions II, III, IV, VIII, IX, X, and XI, comprising genes coding for hypothetical proteins or proteins of unknown functions. Among them, DNA region II, which contains the *ydhB* and *ydhC* genes, was knocked out most frequently, namely, six times. Other DNA regions that underwent frequent mutations were regions III and IV, in which the *yfgQ* and *yveH* genes, each disrupted three times, were identified. Out of 11 regions, only 4 (regions I, V, VI, and VII) comprised genes coding for proteins homologous to proteins with assigned functions, among them the *bglS* gene (region VI, two clones), coding for a putative P-β-glucosidase, and the operon containing genes encoding proteins of the Leloir pathway (region I, 8 clones).

Growth tests of the *ccpA* double mutants. To better characterize the effect of the pGhost9::IS*S1* mutations and to check whether the β-galactosidase-negative phenotype is linked to the expected loss of lactose-fermenting ability, growth of the majority of the *ccpA*-derived double mutants was examined in L-CDM (data not shown). In contrast to expectations, only four out of all the integrants tested displayed a complete inability to grow in this medium. These were mutants with disrupted *galE*, *galT*, or *bglS* genes and a clone containing IS*S1* in the noncoding region upstream of *yebF* (Table 4). Either the other integrants retained full lactose-fermenting ability, or their growth was variously impaired (Table 4).

The roles of the *bglS*, *galK*, *galT*, and *galE* genes were further studied by making stable single-crossover knockouts of each of them. In the same way, the *lacZ* gene, encoding a putative β-galactosidase and the only gene from the Leloir cluster not hit during pGhost9::IS*S1* mutagenesis, was inactivated. Growth of the resulting strains in L-CDM revealed that all of them had the same growth defects as the corresponding pGhost9::IS*S1* integrants. In comparison to *L. lactis* IL1403, the growth of the *lacZ* mutant in CL-CDM was reduced, although the double mutant (*lacZ ccpA*) grew in the same manner as IBB550. The *bglS* single mutant was unable to grow in CL-CDM (data not shown).

The *bglS* mutant has lower P-β-galactosidase activity. Disruption of the *bglS* gene in both IL1403 and IBB550 resulted in an approximately 2.5-fold reduction of P-β-galactosidase activity in G-CDM, compared to their respective parents carrying intact *bglS* (Table 3). The obtained values were low but measurable, indicating that BglS is not the only enzyme with P-β-galactosidase activity in *L. lactis* IL1403.

Since the *bglS* mutants were not able to grow in lactose media, these mutants were also grown in CDM supplemented with galactose, a sugar previously shown to be unable to induce catabolic repression (29). P-β-galactosidase activity was lower in the *bglS* mutant than in IL1403 (Table 3). However, in the presence of galactose together with lactose and inducing amounts of cellobiose, P-β-galactosidase activity reached 51 nmol/min/mg in IL1403 but decreased to 10 nmol/min/mg in the *bglS* mutant. Moreover, the level of P-β-galactosidase activity in galactose-lactose-cellobiose-growing *bglS* mutant cells was decreased in comparison to that in cells grown in a medium containing only galactose. This indicates that no more P-β-galactosidase activity was induced by the presence of lactose and that the obtained value was due to the presence of galactose. These observations are consistent with the complete inability of *bglS* mutant cells to grow in lactose-containing media. In the *bglS* mutants growing in the presence of cellobiose, the P-β-galactosidase activity was reduced approximately 10-fold in comparison to their parental strains, although it was still detectable, reaching a mean level of 24.5 nmol/min/mg. These data suggest that although several genes may encode P-β-galactosidase activity in *L. lactis* IL1403, BglS is the major enzyme involved in lactose hydrolysis.

DISCUSSION

L. lactis IL1403 is a plasmid-cured strain that is unable to assimilate lactose, although after prolonged incubation it begins to utilize this sugar slowly (Fig. 1). Moreover, it was found to be able to assimilate lactose faster after induction by cellobiose (Fig. 1) or in the absence of functional CcpA (Fig. 2). An additional, remarkable feature of this strain is its ability to hydrolyze the lactose analog X-Gal in the presence of cellobiose. These results suggest that a cryptic lactose utilization system exists in *L. lactis* IL1403. The emergence of chromosomally encoded alternative lactose degradation pathways in *Lactococcus* might be a good method for adaptation from the plant environment to milk. Plants are believed to constitute the primary habitat for these bacteria, whereas milk is believed to be a secondary one. Cellobiose, a β-glucoside plant sugar, can serve as a constitutive signal for preadaptation of lactococcal cells to growth in a lactose-containing environment such as milk. Acquiring such a cellobiose-inducible catabolic potential might have provided an opportunity to settle in another environment and gain an evolutionary advantage over other bacteria.

In this article, we characterize the genes involved in both lactose and cellobiose-inducible lactose metabolisms in IL1403 and its *ccpA* mutant, IBB550. By random pGhost9::IS*S1* mutagenesis of IL1403 and IBB550 and screening for inactivation of the β-galactosidase-positive phenotype, several mutants were obtained that had lost their β-galactosidase-positive phenotype but unexpectedly retained the ability to assimilate lactose. It is possible that the affected genes code for proteins that are involved only in X-Gal metabolism or are not involved in sugar metabolism, whereas the β-galactosidase-negative phenotype is caused by side effects (e.g., starvation or a response to environmental changes such as cultivation of mutants at 37°C). It is also possible that the β-galactosidase-negative phenotype is caused by mutations other than those due to pGhost9::IS*S1* integration (e.g., spontaneous mutations in the IL1403 genome). Among all the pGhost9::IS*S1* integrants tested, only insertions of the plasmid in the *lacS*, *bglS*, *galE*, *galT*, *yebF*, and *yheB* genes led to clear inhibition of growth of mutants in cellobiose-lactose- and/or lactose-containing media (Tables 2 and 4). This result strongly suggests that proteins encoded by these genes are directly or indirectly involved in lactose assimilation in *L. lactis* strains. However, it should be taken into account that the physiological effects obtained in the tested mutants might also be due to the polarity of mutations introduced into the downstream genes.

Establishing the inability of the *lacS* mutant to hydrolyze X-Gal appeared to be a highly promising result with respect to understanding lactose catabolism in *L. lactis* IL1403. Indeed, the *lacS* gene is linked to the gene cluster of the Leloir pathway, which, in combination with the chromosomally encoded *lac* genes, has been shown to be engaged in lactose metabolism in several bacteria. Up to now, among *Lactococcus* strains such a permease–β-galactosidase system has been described only for *L. lactis* NCDO2054, a strain fermenting lactose slowly. In *L. lactis* NCDO2054 lactose is transported by a lactose permease-H^+ symporter (26) and is subsequently cleaved by β-galactosidase (53). The fact that this permease has a much higher affinity for galactose than for lactose (26, 47, 48) could explain the poor ability of this strain to grow in lactose-containing media. The genes of the Leloir cluster have not yet been studied in *L. lactis* IL1403, but the predicted genes are also associated with genes needed for lactose assimilation, such as *lacZ* (β-galactosidase) and *lacA* (thiogalactoside acetyltransferase) (4). Furthermore, the *lacS* gene of IL1403 is almost

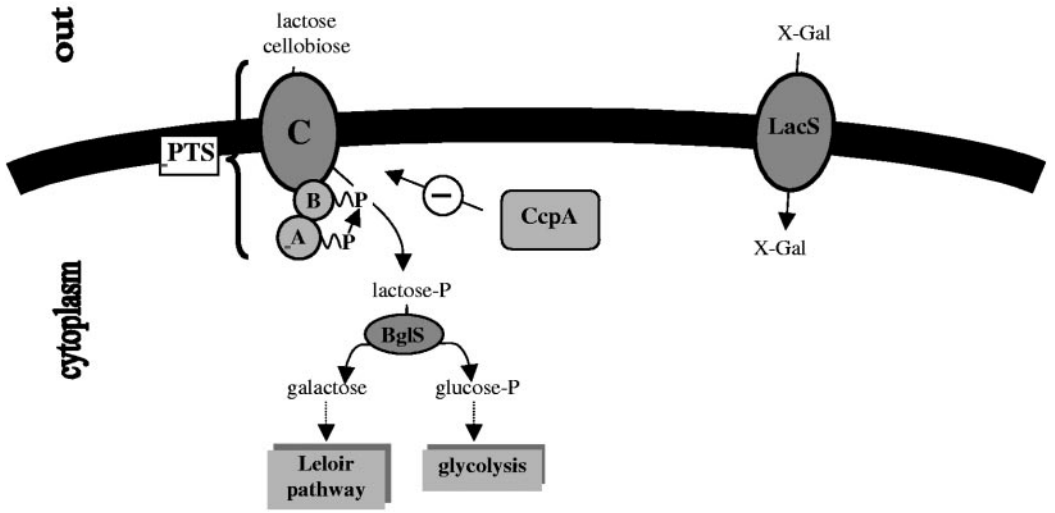

FIG. 3. Schematic representation of the proposed mechanism of lactose- and cellobiose-inducible lactose metabolism in *L. lactis* IL1403. C, PTS β-glucosides-specific component, which also shows affinity for lactose (in IL1403, after induction by cellobiose; in IBB550, without induction) and is repressed by CcpA. A and B, components of the PTS.

identical to that of *L. lactis* NCDO2054 but also to *galP* of the lactose-negative *L. lactis* strain MG1363 (18). Both permeases belong to the same subfamily (TC 2.A.2.2.3 [http://www-biology.ucsd.edu/~msaier/transport/]), which includes permeases specific for galactose transport. However, the MG1363 Leloir gene cluster does not contain genes for β-galactosidase and thiogalactoside acetyltransferase. This might explain why MG1363, despite possessing the same permease as the lactose-positive *L. lactis* NCDO2054, is unable to assimilate lactose.

Since *L. lactis* IL1403 possesses the genes for a complete permease–β-galactosidase system, it seems odd that it is almost unable to assimilate lactose. At least two explanations can be envisaged: (i) lactose transport is inefficient due to low affinity of LacS for lactose or (ii) the strain lacks a functional β-galactosidase. Disruption of *lacS* in IL1403 resulted in the loss of the β-galactosidase-positive phenotype (C-CDM–X-Gal plate) and in a slight reduction of the ability to grow slowly in L-CDM, with no effect on growth in CL-CDM (Table 2). A similar result was observed when *lacS* was disrupted in IBB550; namely, the *lacS ccpA* double mutant also lost its β-galactosidase-positive phenotype (L-CDM–X-Gal plate). However, its growth in lactose-containing media was only slightly reduced in comparison to that of strain IBB550 (Table 4). Moreover, despite the fact that IL1403 possesses a putative LacZ, which is almost identical (98% identity) to the highly expressed LacZ of the lactose-positive *L. lactis* NCDO2054 (53), it does not exhibit β-galactosidase activity.

We therefore postulate that LacS of IL1403 plays a minor role in lactose transfer, both in *L. lactis* IL1403 and in its *ccpA* mutant, and that its function is limited to the transport of X-Gal. Since IL1403 does not exhibit β-galactosidase activity, we also assume that the *lacZ* gene of IL1403 either is inactive or is not expressed. This may explain why IL1403, possessing a full permease–β-galactosidase lactose assimilation genetic system, is a lactose-negative strain.

It is possible that the same system is used for both lactose and cellobiose-inducible lactose assimilation in IBB550, as inactivation of *lacS* in this strain does not affect its lactose fermentation abilities. Since IL1403 lacks the *lac*-PTS genes but possesses several genes encoding putative proteins homologous to β-glucoside-specific PTS permeases (4), we believe that the main lactose uptake system in this strain may be a β-glucoside-dependent PTS. Furthermore, the capability for lactose fermentation in the absence of functional CcpA implies that this PTS is subject to CcpA-mediated CCR. Indeed, an in silico analysis of the IL1403 genome has shown the existence of thousands of putative *cre* sites with various levels of homology to the *cre* consensus (5′-TGNNANCGNTNNCA-3′) (20). This analysis also showed that some consensus *cre* sites are present in the neighborhood of sugar catabolism genes, suggesting the involvement of CcpA in CCR of these genes.

The hypothesis that the main lactose transport system in IL1403 is a β-glucoside-specific PTS is strengthened by the fact that β-galactosidase-negative phenotype mutants were obtained through pGhost9::IS*S1* mutagenesis, in which the *bglS* gene, encoding P-β-glucosidase, had been inactivated. Genes for such hydrolases are mostly associated with operons encoding β-glucoside-specific PTSs, and the products of those genes are involved in the cleavage of C-6-phosphorylated β-glucosides to glucose 6-phosphate and the respective aglycons (45, 49). Indeed, according to the *L. lactis* IL1403 genome sequence (4), *bglS* is directly preceded by the *celB* gene, encoding a predicted protein homologous to the EIIC component of the β-glucoside-specific PEP-PTS. Such a localization suggests the involvement of CelB in lactose uptake in *L. lactis* IL1403.

Since the IL1403 genome does not contain any gene coding for P-β-galactosidase (4), it was surprising to find that this strain is capable of hydrolyzing the C-6-phosphorylated β-galactoside, *o*-nitrophenyl β-D-galactopyranoside-6-phosphate, an analog of lactose-6-P (Table 3). Since there are several genes encoding P-β-glucosidases in *L. lactis* IL1403, the observed P-β-galactosidase activity, induced by cellobiose, might be due to the activity of one of the P-β-glucosidases. This

reaction can be understood, as both P-β-galactosidase and P-β-glucosidase exhibit high sequence similarities that allow them to be classified as belonging to family I of glycosylhydrolases (24). Simons et al. (46) have similarly observed that *L. lactis* mutated in the *lacG* gene (encoding a P-β-galactosidase) is still capable of slow growth on lactose, and this growth has been proposed to likewise depend on P-β-glucosidase activity. Here, we showed that disruption of *bglS* in both IL1403 and IBB550 led to complete inability of the mutants to grow in all lactose-containing media. Additionally, when cultivated in galactose-cellobiose-lactose-containing medium, no more P-β-galactosidase was induced by the presence of lactose in the *bglS* mutant (Table 3). Thus, we postulate that in *L. lactis* IL1403 the metabolisms of β-glucosides (cellobiose) and β-galactosides (lactose) are interconnected and that after internalization by the β-glucoside-specific PTS (presumably involving CelB), lactose-P is hydrolyzed by BglS. Despite the occurrence of several P-β-glucosidase genes in the IL1403 chromosome, it seems that BglS is the major enzyme involved in lactose hydrolysis. Nevertheless, it is not the only enzyme possessing P-β-galactosidase activity. The *bglS* gene is preceded by three *cre* sites, all deviating from the *cre* consensus by two mismatches. We suggest that *bglS* is probably only weakly or not at all regulated by CcpA, because when measured under repressive conditions (in glucose-containing medium), P-β-galactosidase activities in wild-type IL1403 and IBB550 are not very different (Table 3).

Other mutants severely impaired in lactose fermentation were affected in genes of the Leloir pathway. We postulate that lactose-P, internalized by the β-glucoside-specific PTS, is cleaved by BglS into glucose-P and galactose but not to glucose and galactose-P. Then, galactose is further metabolized by the enzymes of the Leloir pathway. The tagatose 6-phosphate pathway, which would deal with the catabolism of galactose-6-P (52), is absent in IL1403 (4).

Based on the results presented above, a putative model of lactose- and lactose-cellobiose-coupled sugar metabolism was made, in which the key elements are the proteins of the β-glucoside-specific PTS (Fig. 3). In the presence of glucose IL1403 is unable to assimilate lactose due to repression by CcpA. Inactivation of *ccpA* results in derepression of the β-glucoside-specific PTS transport system, thus enabling the IL1403 strain to import and grow on lactose. Moreover, the availability of cellobiose activates this PTS transporter, and IL1403 is able to grow on cellobiose and lactose. Internalized lactose is phosphorylated and subsequently split by BglS into galactose and glucose-P, the former of which is further metabolized through the Leloir pathway, while glucose-P enters glycolysis. Based on the results obtained with the *bglS* mutant, which shows a low level of P-β-galactosidase activity, it may be speculated that another P-β-glucosidase is encoded in the IL1403 genome. It is also proposed in this model that internalization of X-Gal occurs independently of the above-described PTS sugar transport system, via LacS.

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REFERENCES

1. **Aleksandrzak, T., M. Kowalczyk, J. Kok, and J. Bardowski.** 2000. Regulation of carbon catabolism in *Lactococcus lactis*. Food Biotechnol. **17:**61–66.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Anderson, D. G., and L. L. McKay.** 1977. Plasmids, loss of lactose metabolism, and appearance of partial and full lactose-fermenting revertants in *Streptococcus cremoris* B1. J. Bacteriol. **129:**367–377.
4. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis ssp. lactis* IL1403. Genome Res. **11:**731–753.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Breidt, F. J., W. Hengstenberg, U. Finkeldei, and G. C. Stewart.** 1987. Identification of the genes for the lactose-specific components of the phosphotransferase system in the *lac*-operon of *Staphylococcus aureus*. J. Biol. Chem. **262:**16444–16449.
7. **Breidt, F. J., and G. C. Stewart.** 1987. Nucleotide and deduced amino acid sequences of the *Staphylococcus aureus* phospho-β-galactosidase gene. Appl. Environ. Microbiol. **53:**969–973.
8. **Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella.** 1984. Two plasmid determine restriction modification systems in *Streptococcus lactis*. Plasmid **11:**260–263.
9. **Cords, B. R., and L. L. McKay.** 1974. Characterization of lactose-fermenting revertants from lactose-negative *Streptococcus lactis* C2 mutants. J. Bacteriol. **119:**830–839.
10. **Crow, V. L., and T. D. Thomas.** 1984. Properties of a *Streptococcus lactis* strain that ferments lactose slowly. J. Bacteriol. **157:**28–34.
11. **Delorme, C., S. D. Ehrlich, and P. Renault.** 1999. Regulation of expression of the *Lactococcus lactis* histidine operon. J. Bacteriol. **181:**2026–2037.
12. **de Vos, W. M., and G. Simons.** 1988. Molecular cloning of lactose genes in dairy lactic streptococci: the phospho-β-galactosidase and β-galactosidase genes and their expression products. Biochimie **70:**461–473.
13. **de Vos, W. M., and E. E. Vaughan.** 1994. Genetics of lactose utilization in lactic acid bacteria. FEMS Microbiol. Rev. **15:**217–237.
14. **de Vos, W. M., I. Boerrigter, R. J. Van Rooyen, B. Reiche, and W. Hengstenberg.** 1990. Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. J. Biol. Chem. **265:**22554–22560.
15. **Fath, M. J., and R. Kolter.** 1993. ABC exporters. Microbiol. Rev. **57:**995–1017.
16. **Fujita, Y., Y. Miwa, A. Galinier, and J. Deutscher.** 1995. Specific recognition of the *Bacillus subtilis* gut *cis*-acting catabolite-responsive element by a protein complex formed between CcpA and seryl-phosphorylated HPr. Mol. Microbiol. **17:**953–960.
17. **Gibson, T. J.** 1984. Studies on the Epstein-Barr virus genome. Ph.D. thesis. University of Cambridge, Cambridge, England.
18. **Grossiord, B. P., E. J. Luesink, E. E. Vaughan, A. Arnaud, and W. M. de Vos.** 2003. Characterization, expression, and mutation of the *Lactococcus lactis galPMKTE* genes, involved in galactose utilization via the Leloir pathway. J. Bacteriol. **185:**870–878.
19. **Grundy, F. J., A. J. Turinsky, and T. M. Henkin.** 1994. Catabolite regulation of *Bacillus subtilis* acetate and acetoin utilization genes by CcpA. J. Bacteriol. **176:**4527–4533.
20. **Guédon, E., E. Jamet, and P. Renault.** 2001. Gene regulation in *Lactococcus lactis*: the gap between predicted and characterized regulators. Antonie Leeuwenhoek **82:**93–112.
21. **Hayes, F., G. F. Fitzgerald, W. M. de Vos, and C. Daly.** 1991. Molecular organization of the minimal replicon of novel, narrow-host-range, lactococcal plasmid pCI305. Plasmid **25:**16–26.
22. **Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss.** 1991. Catabolite repression of alpha-amylase gene expression in *Bacillus subtilis* involves a trans-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:**575–584.
23. **Henkin, T. M.** 1996. The role of CcpA transcriptional regulator in carbon metabolism in *Bacillus subtilis*. FEMS Microbiol. Lett. **135:**9–15.
24. **Henrissat, B.** 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. **280:**309–316.
25. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Mirobiol. **55:**3119–3123.
26. **Kashket, E. R., and T. H. Wilson.** 1973. Proton-coupled accumulation of galactoside in *Streptococcus lactis* 7962. Proc. Natl. Acad. Sci. USA **70:**2866–2869.
27. **Konings, W. N., J. S. Lolkema, and B. Poolman.** 1995. The generation of metabolic energy by solute transport. Arch. Microbiol. **164:**235–242.

28. **Leenhouts, K., G. Buist, A. Bolhuis, A. Ten Berge, J. Kiel, I. Mierau, M. Dabrowska, G. Venema, and J. Kok.** 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol. Gen. Genet. **253:**217–224.
29. **Luesink, E. J., R. E. M. A. van Herpen, B. P. Grossiord, O. P. Kuipers, and W. M. de Vos.** 1998. Transcriptional activation of the glycolytic *las* operon and catabolite repression of the *gal* operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. Mol. Microbiol. **30:**789–798.
30. **Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss.** 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. J. Bacteriol. **178:**931–935.
31. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Miwa, Y., M. Saikawa, and Y. Fujita.** 1994. Possible function and some properties of the CcpA protein of Bacillus subtilis. Microbiology **140:** 2567–2575.
33. **Miwa, Y., A. Nakata, A. Ogiwara, M. Yamamoto, and Y. Fujita.** 2000. Evaluation and characterization of catabolite-responsive elements (cre) of Bacillus subtilis. Nucleic Acids Res. **28:**1206–1210.
34. **Moreno, M. S., B. L. Schneider, R. R. Maile, W. Weyler, and M. H. Saier, Jr.** 2001. Catabolite repression mediated by the CcpA protein in *Bacillus subtilis*: novel modes of regulation revealed by whole-genome analyses. Mol. Microbiol. **39:**1366–1381.
35. **Otto, R., Ten Bring, B., Veldcamp, H., and W. N. Konings.** 1983. The relations between growth rate and electrochemical proton gradient on *Streptococcus cremoris*. FEMS Microbiol. Lett. **16:**69–74.
36. **Poolman, B.** 1990. Precursor/product antiport in bacteria. Mol. Microbiol. **4:**1629–1636.
37. **Poolman, B.** 1993. Energy transduction in lactic acid bacteria. FEMS Microbiol. Rev. **12:**125–148.
38. **Poolman, B., T. J Royer, S. E Mainzer, and B. F. Schmidt.** 1989. Lactose transport system of *Streptococcus thermophilus*: a hybrid protein with homology to the melibiose carrier and enzyme III of phosphoenolpyruvate-dependent phosphotransferase systems. J. Bacteriol. **171:**244–253.
39. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolopyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543–594.
40. **Raya, R., J. Bardowski, P. S. Andersen, S. D. Ehrlich, and A. Chopin.** 1998. Multiple transcriptional control of the *Lactococcus lactis trp* operon. J. Bacteriol. **180:**3174–3180.
41. **Rosey, E. L., and G. C. Stewart.** 1992. Nucleotide and deduced amino acid sequences of the *lacR*, *lacABCD*, and *lacFE* genes encoding the repressor, tagatose 6-phosphate gene cluster, and sugar-specific phosphotransferase system components of the lactose operon of *Streptococcus mutans*. J. Bacteriol. **174:**6159–6170.
42. **Rosey, E. L., B. Oskouian, and G. C. Stewart.** 1991. Lactose metabolism by *Staphylococcus aureus:* characterization of *lacABCD*, the structural genes of the tagatose 6-phosphate pathway. J. Bacteriol. **173:**5992–5998.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
45. **Schnetz, K., C. Toloczyki, and B. Rak.** 1987. β-Glucoside (*bgl*) operon of *Escherichia coli*: nucleotide sequence, genetic organization, and possible evolutionary relationship to regulatory components of two *Bacillus subtilis* genes. J. Bacteriol. **169:**2579–2590.
46. **Simons, G. F. M., M. Nijhuis, and W. M. de Vos.** 1993. Integration and gene replacement in the *Lactococcus lactis lac* operon: induction of a cryptic phospho-β-glucosidase in LacG-deficient strains. J. Bacteriol. **175:**5168–5175.
47. **Thompson, J.** 1979. Lactose metabolism in *Streptococcus lactis*: phosphorylation of galactose and glucose moieties in vivo. J. Bacteriol. **140:**774–785.
48. **Thompson, J.** 1980. Galactose transport systems in *Streptococcus lactis*. J. Bacteriol. **144:**683–691.
49. **Tobisch, S., P. Glaser, S. Krüger, and M. Hecker.** 1997. Identification and characterization of a new β-glucoside utilization system in *Bacillus subtilis*. J. Bacteriol. **179:**496–506.
50. **Turinsky, A. J., F. J. Grundy, J. H. Kim, G. H. Chambliss, and T. M. Henkin.** 1998. Transcriptional activation of the *Bacillus subtilis ackA* gene requires sequences upstream of the promoter. J. Bacteriol. **180:**5961–5967.
51. **van Rooijen, R. J., and W. M. de Vos.** 1990. Molecular cloning, transcriptional analysis, and nucleotide sequence of *lacR*, a gene encoding the repressor of the lactose phosphotransferase system of *Lactococcus lactis*. J. Biol. Chem. **265:**18499–18503.
52. **van Rooijen, R. J., S. van Schalkwijk, and W. M. de Vos.** 1991. Molecular cloning, characterization, and nucleotide sequence of the tagatose 6-phosphate pathway gene cluster of the lactose operon of *Lactococcus lactis*. J. Biol. Chem. **266:**7176–7181.
53. **Vaughan, E. E., R. D. Pridmore, and B. Mollet.** 1998. Transcriptional regulation and evolution of lactose genes in the galactose-lactose operon of *Lactococcus lactis* NCDO2054. J. Bacteriol. **180:**4893–4902.
54. **Weickert, M. J., and G. H. Chambliss.** 1990. Site-directed mutagenesis of a catabolite repressor operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**6238–6242.
55. **Weickert, M. J., and S. Adhya.** 1992. A family of bacterial regulators homologous to Gal and Lac repressors. J. Biol. Chem. **267:**15869–15874.
56. **Wells, J. M., P. M. Wilson, and R. W. F. Le Page.** 1993. Improved cloning vectors and transformation procedure for *Lactococcus lactis*. J. Appl. Bacteriol. **74:**629–636.
57. **Williams, S. G., J. A. Greenwood, and C. W. Jones.** 1992. Molecular analysis of the *lac* operon encoding the binding-protein-dependent lactose transport system and β-galactosidase in *Agrobacterium radiobacter*. Mol. Microbiol. **6:**1755–1768.